INFECTION AND IMMUNITY, June 2009, p. 2557–2567 Vol. 77, No. 6
0019-9567/09/$08.00+0 doi:10.1128/IAI.01481-08

Protection of *Mycobacterium tuberculosis* from Reactive Oxygen Species Conferred by the *mel2* Locus Impacts Persistence and Dissemination▽

Suat L. G. Cirillo,[1] Selvakumar Subbian,[1] Bing Chen,[2] Torin R. Weisbrod,[2] William R. Jacobs, Jr.,[2] and Jeffrey D. Cirillo[1]*

Department of Microbial and Molecular Pathogenesis, Texas A&M Health Sciences Center, 467 Reynolds Medical Building, College Station, Texas 77843,[1] and Howard Hughes Medical Institute, Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461[2]

Received 3 December 2008/Returned for modification 31 January 2009/Accepted 28 March 2009

Persistence of *Mycobacterium tuberculosis* in humans represents a major roadblock to elimination of tuberculosis. We describe identification of a locus in *M. tuberculosis*, *mel2*, that displays similarity to bacterial bioluminescent loci and plays an important role during persistence in mice. We constructed a deletion of the *mel2* locus and found that the mutant displays increased susceptibility to reactive oxygen species (ROS). Upon infection of mice by aerosol the mutant grows normally until the persistent stage, where it does not persist as well as wild type. Histopathological analyses show that infection with the *mel2* mutant results in reduced pathology and both CFU and histopathology indicate that dissemination of the *mel2* mutant to the spleen is delayed. These data along with growth in activated macrophages and infection of Phox$^{-/-}$ and iNOS$^{-/-}$ mice and bone marrow-derived macrophages suggest that the primary mechanism by which *mel2* affects pathogenesis is through its ability to confer resistance to ROS. These studies provide the first insight into the mechanism of action for this novel class of genes that are related to bioluminescence genes. The role of *mel2* in resistance to ROS is important for persistence and dissemination of *M. tuberculosis* and suggests that homologues in other bacterial species are likely to play a role in pathogenesis.

Despite extensive efforts to eradicate tuberculosis, caused by *Mycobacterium tuberculosis*, worldwide and prevent the spread of antibiotic-resistant strains, tuberculosis remains one of the most frequent causes of death in humans. Currently, one-third of the world's population is thought to be persistently infected with tuberculosis (6, 19, 20). A better understanding of the mechanisms that lead to persistence in humans is needed before it will be possible to develop rational strategies to prevent establishment of latency and block reactivation from it. Although the mouse model, even when infected by the natural low-dose aerosol route, does not replicate all aspects of pathogenesis by tuberculosis, its cost-effectiveness and the presence of numerous reagents make it an important tool for examination of the acute and persistent stages of infection (26, 50, 51). As a result, much of our knowledge regarding the role of the host in controlling infections as well as the bacterial factors involved has been obtained using the mouse model (26, 31, 34).

Initially, tubercle bacilli encounter naïve alveolar macrophages in the mouse lung that produce low levels of reactive oxygen species (ROS) and undergo an oxidative burst in response to infection (8, 52). In the absence of ROS production, there is a modest transient advantage for tuberculosis in the lung, suggesting that ROS initially assist in the control of bacterial growth (16). This innate immune response does little to prevent the growth of the bacteria in mouse lungs, since for the first month after infection, bacterial numbers increase to a million or more. This time point corresponds to the peaks in the numbers of CD4 and CD8 T cells in the lung (38), maximal tumor necrosis factor alpha (TNF-α) levels (5, 32), and the initiation of a strong cell-mediated immune response. The cell-mediated immune response leads to control of bacterial growth, higher gamma interferon (IFN-γ) levels and tuberculosis persistence at stable numbers for between 1 and 2 years (38) before reactivation, most likely the result of a weakened immune system due to the age of the mice (49). TNF-α (44, 57) and IFN-γ (15, 27) levels are important for controlling tuberculosis growth and maintenance of the persistent state in the mouse model of infection. One of the reasons that TNF-α and IFN-γ help to control tuberculosis is that they provide signals for the production of both ROS and reactive nitrogen species (RNS) by macrophages (8, 25, 28, 32, 40, 65). Although *M. tuberculosis* is considered relatively resistant to ROS, mutations that impact those bacterial pathways involved in resistance can affect virulence (21, 33, 39, 47, 53). In contrast to ROS, RNS, produced by the nitric oxide synthase (iNOS) that is induced by IFN-γ, inhibit the growth of mycobacteria and are critical to maintenance of the persistent state in mice (7, 8, 22, 27, 29, 42). Although the role of RNS in the control of *M. tuberculosis* growth in mice seems well proven, the involvement of RNS in human (58) and in other animal, including guinea pig (36, 68), tuberculosis models is less clear, possibly because the presence of NO has been difficult to demonstrate (9, 24, 46, 54, 67). These observations suggest that *M. tuberculosis* genes involved in resistance to either ROS or RNS will play a role in persistence.

We recently identified a locus in *Mycobacterium marinum*, designated *mel2*, that affects resistance to ROS and RNS (61, 62). Interestingly, this locus has three genes, *melF*, *melG*, and *melH*, with similarity to the *luxA*, *luxG*, and *luxH* biolumines-

* Corresponding author. Mailing address: Dept. of Microbial and Molecular Pathogenesis, Texas A&M Health Sciences Center, 467 Reynolds Medical Building, College Station, TX 77843. Phone: (979) 458-0778. Fax: (979) 845-3479. E-mail: jdcirillo@medicine.tamhsc.edu.

▽ Published ahead of print on 6 April 2009.

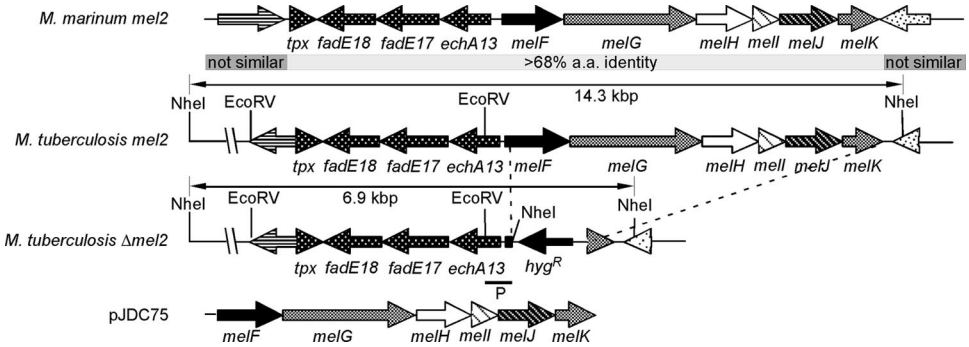

FIG. 1. Organization and similarity of the *mel2* locus in *M. marinum* and *M. tuberculosis* as well as the structure of the *mel2* deletion mutant and the single-copy, site-specific integrating plasmid (pJDC75) used for the complementation of the *mel2* mutant. The identity at the amino acid level between *M. marinum* and *M. tuberculosis* for each open reading frame within this locus is >68%, whereas the adjoining regions display no similarity between these two mycobacterial species (shown by the gray bar). Arrows indicate the deduced direction of transcription and length of each open reading frame. The distance between the two NheI sites producing the 14.3-kbp fragment in wild-type *M. tuberculosis* and the 6.9-kbp fragment in the *mel2* deletion mutant observed by Southern analysis is indicated by the double arrow line. The position of the Hyg^r gene is indicated. Dashed lines indicate the positions of the deletion produced by allelic exchange. The line designated P indicates the position of the probe used for Southern analyses.

cence genes, respectively, in other bacterial species (23). The luciferase (*luxA*) genes in other organisms can affect resistance to ROS (41, 55, 66) in addition to their role in bioluminescence. Bacterial luciferases are thought to scavenge H_2O_2 in a catalase-like reaction in which the enzyme-bound flavin mononucleotide (FMN) hydroperoxide and H_2O_2 decompose, releasing water and oxygen, to the enzyme-bound hydroxy-FMN that spontaneously decomposes to water, light, and enzyme-bound FMN (66). These observations suggest the intriguing possibility that the *mel2* locus will be important for tuberculosis pathogenesis, since *M. tuberculosis* must resist ROS produced by the host immune response during infections. In order to test this hypothesis, we isolated an *M. tuberculosis* mutant in the *mel2* locus and confirmed the loss of *mel2* activity by an increase in ROS susceptibility. The *mel2* mutant was evaluated for its role in virulence using the mouse model and macrophage infection in vitro. Mice and macrophages deficient in ROS and RNS were used to probe the primary host defense mechanisms that the *mel2* locus is necessary to protect tuberculosis against. We found that the *mel2* mutant displays increased susceptibility to ROS and is defective for growth in activated macrophages. Our data also suggest that the *mel2* locus plays an important role during the persistence and dissemination of *M. tuberculosis*. The primary mechanism by which *mel2* affects these events appears to be resistance to ROS. Overall, these studies provide evidence for the role of bioluminescence-related genes, such as *mel2*, in protecting *M. tuberculosis*, and most likely other bacterial species that carry similar genes, against ROS.

MATERIALS AND METHODS

Strains and growth conditions. *M. tuberculosis* strain Erdman (ATCC 35801) and derivatives were grown in 7H9 broth (Difco, Detroit, MI) supplemented with 0.5% glycerol, 10% OADC (oleic acid dextrose complex without catalase), and 0.05% Tween 80 (M-OADC-TW broth) or Middlebrook 7H9 supplemented with 10% OADC and 15 g/liter Bacto agar (M-OADC agar) or on 7H11 selective agar (Difco). The composition of the OADC supplement used is identical to that described in the Difco manual (18) with the exception that it does not contain catalase. A stock solution of 1% (wt/vol) oleic acid is made in 0.2 N NaOH prior to adding 5 ml of the stock solution per 100 ml final volume of the OADC supplement. Prior to use in assays, aliquots of liquid cultures are taken into sealed tubes and vortexed vigorously for 2 min followed by centrifugation at 2,000 × *g* for 1 s. Samples of bacteria are taken from the top half of the resulting culture to ensure that single-cell suspensions with few clumps of bacteria are used for assays. When necessary media and plates were supplemented with 50 μg/ml hygromycin and 25 μg/ml kanamycin. Frozen stocks were prepared from strains for experiments by growth standing at 37°C until an optical density at 600 nm of 0.5 and stored in aliquots at −80°C until use. *Mycobacterium smegmatis* strain mc^{2}155 cultures were grown in M-OADC-TW (23) for 3 days at 37°C prior to use for the propagation of shuttle phasmids.

Cell lines, primary cells, and culture conditions. The murine macrophage cell line J774A.1 (ATCC TIB67) was maintained at 37°C and 5% CO_2 in high glucose Dulbecco's modified Eagle medium (DMEM; Gibco, Bethesda, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and 2 mM L-glutamine. Mouse bone marrow-derived macrophages (BMDM) were collected from the femurs, tibiae, and humeri of infected mice as described previously (56). Briefly, the marrow was flushed out with cold sterile phosphate-buffered saline (PBS), and cells were collected by centrifugation at 800 rpm at room temperature. The cell pellets were washed twice with ACK buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, 0.01 mM Na_2EDTA), followed by an additional wash with RPMI (Gibco) supplemented with 10% heat-inactivated FBS, 1% L-glutamine, 5% L-929 supernatant, and 1% penicillin/streptomycin (Mediatech, Inc.). The cells were suspended in RPMI containing 30% L-929 supernatant and allowed to differentiate for 7 days to obtain BMDM. Human peripheral blood monocyte-derived macrophages (PBMC) were isolated as described previously (10, 11). Basically, PBMC were isolated from 50 ml of human blood obtained from healthy volunteers. The mononuclear cell fraction was purified by centrifugation in Ficoll at 700 × *g* for 30 min. The PBMC band was removed, washed twice, and suspended in RPMI plus 0.1% heat-inactivated human serum to 10^6 cells/ml. Cells were seeded in 24-well dishes and incubated for 2 h at 37°C. Nonadherent cells were removed by washing, and experiments were carried out in RPMI plus 5% heat-inactivated human serum.

Construction and complementation of the Δ*mel2* mutant. The Δ*mel2* mutant *M. tuberculosis* strain was constructed by allelic exchange using the shuttle phasmid phAE87 as described previously (1, 30). Basically, a 7,034-bp region beginning 60 bp from the putative translational start of *melF*, the first gene in *mel2*, and ending 30 bp from the translational stop of *melK*, the last gene in *mel2*, was replaced with a hygromycin resistance (Hyg^r) cassette. The presence of the appropriate mutation in the chromosome of *M. tuberculosis* was confirmed by Southern blot analyses using the probe indicated in Fig. 1 and PCR from within the Hyg^r cassette into the *melF* upstream sequence and within the deleted region. Southern blot analyses identified a 14.3-kbp NheI fragment in wild-type *M. tuberculosis* and a 6.9-kbp NheI fragment in the Δ*mel2* mutant, as expected.

The complementation of the Δ*mel2* mutant was achieved using a single-copy, site-specific integrating construct that carries the endogenous promoter for *mel2*. This complementing construct, designated pJDC75 (Fig. 1), was made by cloning the entire *mel2* locus along with 181 bp upstream of the translational start for

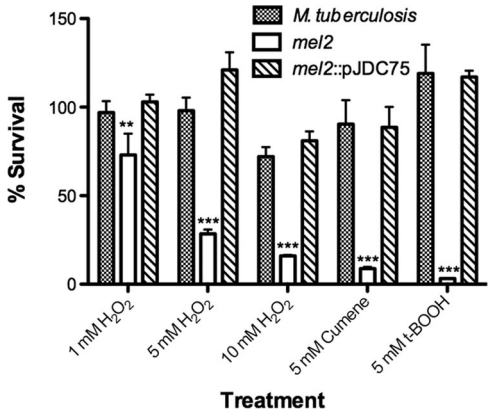

FIG. 2. Susceptibility of the *mel2* mutant to H_2O_2, t-BOOH, and cumene peroxide (Cumene). The percentage of viable *M. tuberculosis* bacteria was determined by measuring bacterial CFU after a 30-min treatment with each compound. The percentage of survival is calculated as (CFU T_{30}/CFU T_0) × 100, where CFU T_{30} is the number of CFU at the 30-min time point and CFU T_0 is the number of CFU at the 0-min time point. Three asterisks indicate a P of <0.001 and two asterisks indicate a P of <0.01, which are significantly different from that for wild-type *M. tuberculosis* under the same treatment conditions.

melF, the putative promoter region for *mel2*, into the NheI site in the L5 attachment site integrating vector pYUB178, which carries a kanamycin resistance gene (61). The Δ*mel2* mutant was transformed with pJDC75 and transformants selected by plating on kanamycin and Hyg. The presence of pJDC75 in the *mel2* mutant was confirmed by PCR and by the ability to confer wild-type resistance to ROS (Fig. 2).

Determination of ROS susceptibility. The susceptibility of *M. tuberculosis* to ROS was determined essentially as described previously (62). Basically, *M. tuberculosis* strains were incubated at a concentration of 10^6 CFU/ml in 1 mM, 5 mM, or 10 mM H_2O_2; 5 mM tert-butyl hydroperoxide (t-BOOH); or 5 mM cumene peroxide in M-OADC-TW for 30 min at 37°C. The numbers of viable bacteria were determined before and after treatment to measure the percentage survival for each strain by plating dilutions on M-OADC agar.

Growth assays in macrophages. Growth of *M. tuberculosis* in mouse and human macrophages was evaluated in J774A.1 cells, BMDM, and PBMC in a manner similar to that described previously (61). J774A.1 cells were seeded at 2.5×10^5 cells/well in 24-well tissue culture plates and incubated overnight (ON) at 37°C in DMEM. In the case of the activated cells, the medium was changed, 100 U murine IFN-γ was added, and the cells were incubated ON at 37°C. The medium was then changed again, 1 μg/ml lipopolysaccharide (LPS) was added, and the cells were incubated ON at 37°C. The medium was then changed just prior to infection with 0.2 ml that contains 10^5 bacteria, equivalent to a multiplicity of infection of approximately 0.1 (bacteria/cell). The bacteria were incubated with the cells for 30 min at 37°C and washed twice with warm PBS, and the medium was replaced with DMEM plus 1% FBS. The cells in a triplicate set of wells were lysed at each time point for each strain with 0.1% Triton X-100. Dilutions were made and plated on M-OADC agar plates to determine the number of viable bacteria present. The medium on the cells was replaced every 3 days throughout the course of the long-term growth experiments. The BMDM were treated in the same manner as the J774A.1 cells, except that they were directly seeded at 1×10^6 cells/well and incubated overnight prior to activation with 100 U IFN-γ in RPMI medium. Human PBMC were used in a similar manner; they were seeded at 1×10^6 cells/well and incubated overnight prior to activation with 40 U human IFN-γ in RPMI medium. Cell viability throughout all the experiments was >90% and did not appear to play a role in the differences observed for *M. tuberculosis* growth under these conditions.

Measurement of ROS and RNS levels. Levels of ROS and RNS produced by macrophages were determined as described previously (61). Basically, BMDM were stimulated with 100 U mouse IFN-γ plus 1 μg LPS or 10^5 *M. tuberculosis* cells for 24 h at 37°C. ROS levels were evaluated by measuring H_2O_2 production with the horseradish peroxidase-dependent oxidation of phenol red and by comparing the levels to a standard curve developed with known concentrations of H_2O_2. The levels of RNS were determined by measuring the NO_2^- content through a reaction with Griess reagent and comparing the levels to a standard curve constructed using $NaNO_3$ to determine the actual concentration of NO_2^-.

Measurement of *M. tuberculosis* LPO levels. Lipid hydroperoxides (LPO) were measured in the *M. tuberculosis* cells by extraction into chloroform and direct quantification using the LPO assay kit (Cayman Chemical) as recommended by the manufacturer. A total of 10^6 CFU of each bacterial strain were treated with either 1 mM H_2O_2 or 10 mM t-BOOH for 16 h at 37°C. The bacteria were mixed with an equal volume of a saturated methanolic solution of extract R and extracted with 2 volumes of ice-cold chloroform. Dilutions were made with chloroform-methanol (2:1, vol/vol) and aliquots analyzed with thiocyanate as the chromogen by reading absorbance at 500 nm. The concentrations of LPO in each sample were then determined using a standard curve constructed using 13-hydroperoxy-octadecadienoic acid as a standard.

Low-dose aerosol infection of mice. Five- to seven-week-old female C57BL/6, inducible nitric oxide synthase negative (iNOS$^{-/-}$), and NADPH oxidase subunit gp91 negative (Phox$^{-/-}$) mice were obtained from Jackson Laboratories. All animals were housed in polycarbonate microisolator cages in a temperature-, humidity-, and light-controlled environment. There were five animals in each experimental group. Animals were assigned randomly to experimental groups, allowed to acclimate to the facilities for 1 week and fed commercial chow and tap water ad libitum. Low-dose infections were carried out by the aerosol route to deliver between 10 and 50 CFU to the lungs using a Madison chamber aerosol generation device, in a manner similar to that described previously (13). Portions of the lungs and spleens were homogenized in PBS and dilutions plated on 7H11 selective medium to determine the numbers of bacteria present at each time point.

Histopathological analyses. Histopathology was carried out to evaluate the pathology of *M. tuberculosis*-infected mice essentially as described previously (12). Basically, portions of the lungs and spleens were fixed in 10% neutral buffered formalin for embedding in paraffin, sectioning at 5 μm, and staining with either hematoxylin and eosin or acid-fast stain. A blinded examination of at least three serial sections from each mouse was carried out to evaluate the numbers of granulomas, the presence of acid-fast bacilli, the degree of mixed cell infiltrate, and inflammation.

Statistical analyses. All experiments were carried out in triplicate and repeated at least three times, except where specifically noted. The significance of the results was determined using the Student's t test or analysis of variance, as needed. P values of <0.05 were considered significant.

RESULTS

Analysis of the *mel2* locus and identification of the *M. tuberculosis* genes. The deduced amino acid sequences of the six putative genes within the *M. marinum mel2* locus, *melF* to *melK*, were each individually used to search the *M. tuberculosis* genome for similar genes. A region of 11,122 bp was identified in the *M. tuberculosis* genome where the six genes within the *mel2* locus are in the same arrangement as in the *M. marinum* chromosome (23). The entire region, from Rv1936 to Rv1941 and four putative genes upstream of the *mel2* locus, has high similarity to the *M. marinum* chromosome, with greater than 68% identity compared to the deduced amino acid sequences of all 10 genes and exactly the same gene order and deduced direction of transcription (Fig. 1). These observations suggest that the function of all 10 of the putative genes within this region is evolutionarily conserved between *M. marinum* and *M. tuberculosis* and that their functions may be related to each other. Interestingly, through the course of these analyses, we identified another locus that is present in both *M. marinum* and *M. tuberculosis* but has only two genes, Rv3618 and Rv3617, similar to the *melF* and *melH* genes in *mel2*, respectively. The *melH* gene is an epoxide hydrolase, also known as *ephB*, and its homologue, Rv3617, is annotated as *ephA* in the *M. tuberculosis* genome. The *melF* gene and its homologue, Rv3618, are both annotated as hypothetical proteins similar to LuxA-related putative monooxygenase. We suggest that an appropriate designation of Rv3618 is *melF2* and that the *melF* gene located

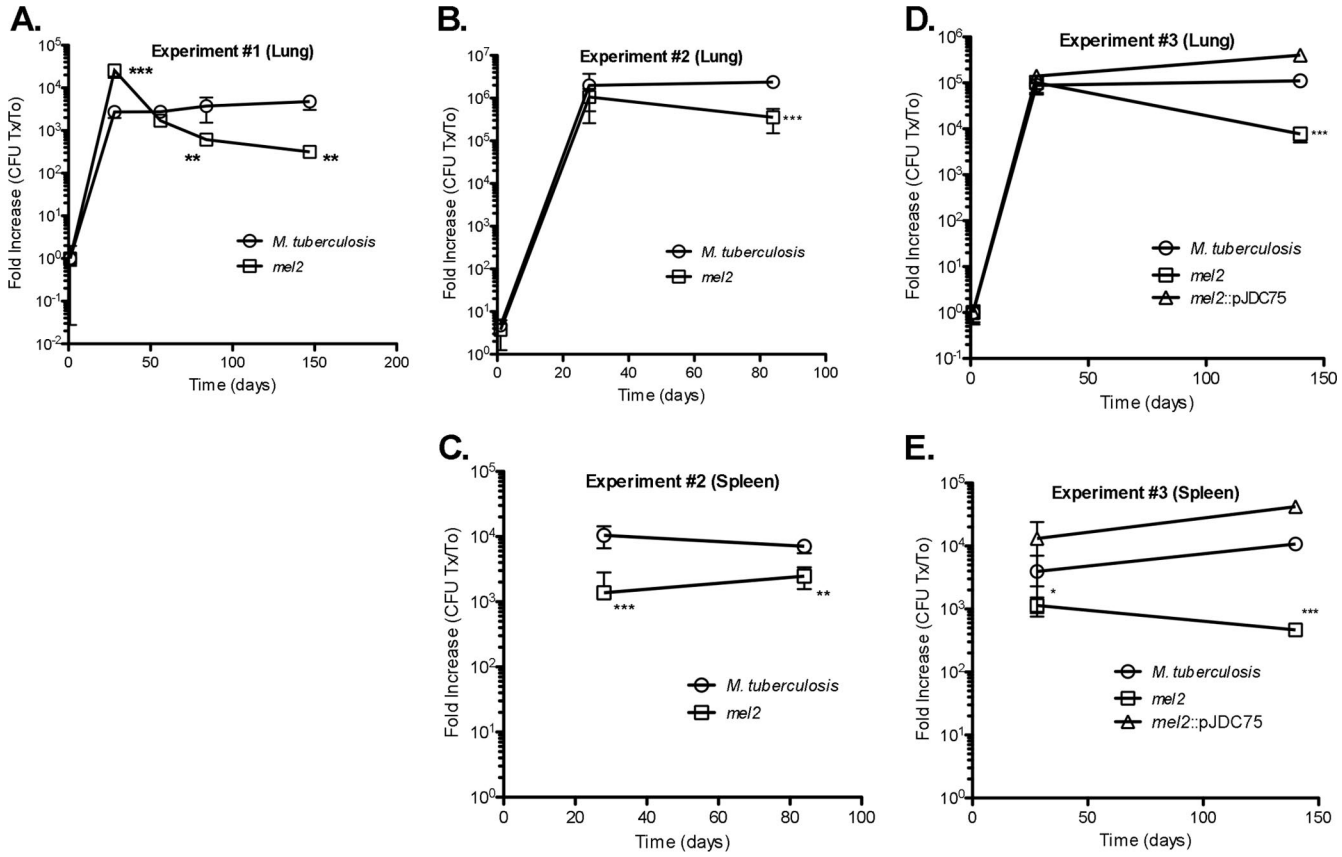

FIG. 3. Growth and persistence of *mel2* mutant in C57BL/6 mice. Data shown are for three experiments with five animals in each group for each experiment. The complementing strain (*mel2*::pJDC75) contains the complete *mel2* locus integrated site specifically in a single copy on the chromosome. The increase is calculated for both the lung (A, B and D) and spleen (C and E) based on the initial numbers of bacteria that seeded the lungs on day 1 after low-dose aerosol infection. One asterisk indicates a P of <0.05, two asterisks indicate a P of <0.01, and three asterisks indicate a P of <0.001, which are significantly different from that for wild-type *M. tuberculosis* in the same organ.

in the *mel2* locus could be designated *melF1*. A search of the available sequence data at NCBI resulted in the identification of 47 bacterial genera that carry genes similar to *melF* that are annotated as encoding hypothetical proteins. Interestingly, more than 70% of these bacteria are known pathogens (49%) or symbionts (21%), suggesting that *melF* may play a role during bacterial interactions with eukaryotes. These observations suggest that information regarding the function of *melF* in *M. tuberculosis* is relevant to genes of unknown function that are widespread in bacterial species, particularly pathogens and symbionts.

Construction and characterization of the *M. tuberculosis mel2* mutant. Phage transduction was used to construct a deletion of the entire *mel2* locus in *M. tuberculosis* (Fig. 1). The presence of the appropriate mutation in the chromosome was confirmed by PCR and Southern analyses (data not shown). The *mel2* deletion mutant did not display a defect in growth or any difference in clumping in laboratory medium compared to wild-type *M. tuberculosis* (data not shown). A single-copy integrating construct, pJDC75, that carries the entire *mel2* locus and its promoter was used to complement the Δ*mel2* mutation. The transformation of pJDC75 into the Δ*mel2* mutant was confirmed by PCR prior to conducting further studies with the wild-type, mutant, and complementing strains in parallel (data not shown). As expected, the *M. tuberculosis* Δ*mel2* mutant is more susceptible to H_2O_2 ($P < 0.001$) than the wild type (Fig. 2), suggesting that the *M. tuberculosis mel2* locus plays a role in the defense against ROS. Data obtained with other ROS-generating compounds, cumene hydroperoxide and t-BOOH, were similar to those obtained with H_2O_2. Thus, we have successfully deleted the entire *M. tuberculosis mel2* locus, confirmed the structure of this mutated region, and obtained evidence that this mutation affects susceptibility to ROS.

***mel2* plays a role in persistence in C57BL/6 mice.** Since bioluminescence pathways in other bacteria can affect susceptibility to ROS and the *mel2* mutation affects resistance to ROS, it is likely that *mel2* will also affect virulence. We evaluated the role of *mel2* in virulence using the low-dose (<100 bacteria per lung) aerosol infection model for tuberculosis in C57BL/6 mice. We found that during the first 4 weeks postinfection, the *mel2* mutant displays higher ($P < 0.001$) bacterial numbers in the lung compared to those of wild-type *M. tuberculosis* (Fig. 3). However, the *mel2* mutant does not persist as well as the wild type and displays significantly ($P < 0.01$) lower numbers of bacteria in the lung at 84 to 147 days postinfection. Although these results were intriguing, we were concerned that, in experiment 1, the infectious dose with the *mel2* mutant was inadvertently more than sixfold higher than that for the

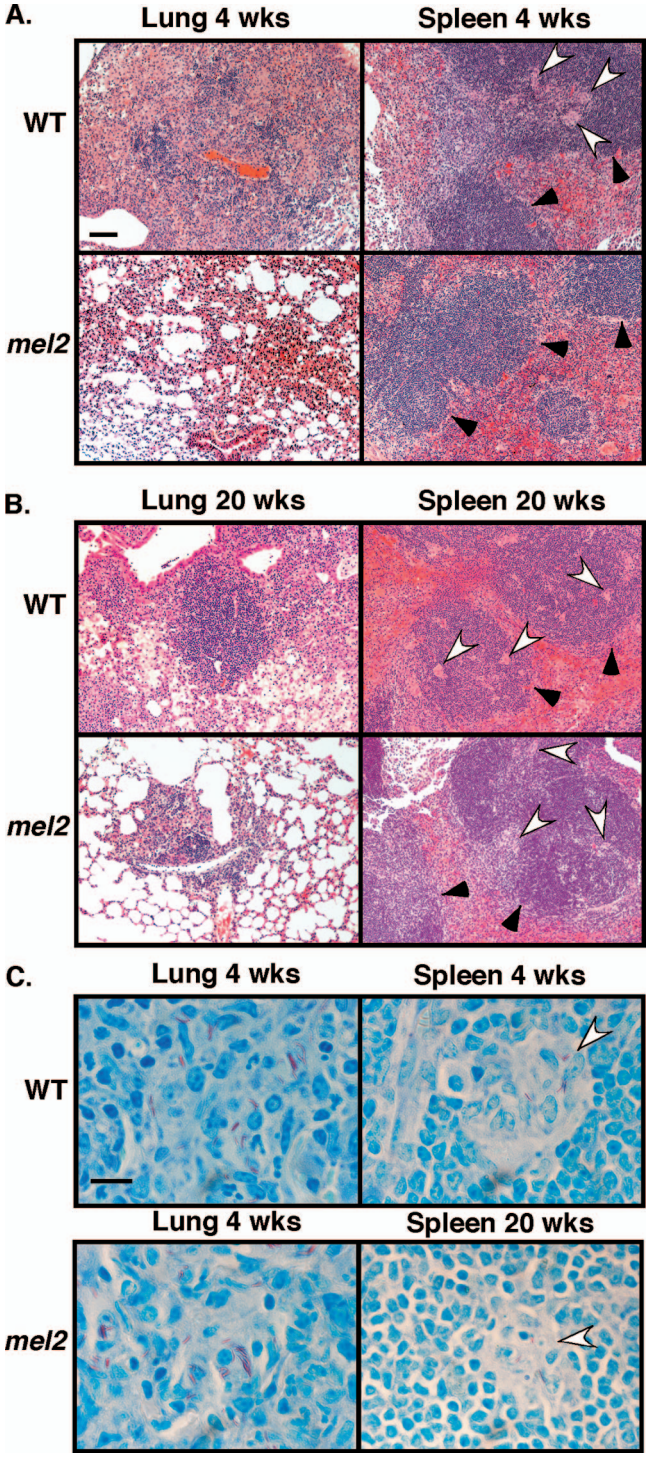

FIG. 4. Histopathological changes in the lungs and spleens of mice infected with wild type (WT) and *mel2 M. tuberculosis*. Hematoxylin and eosin stains were use to evaluate the pathology in tissues from experiment 2 (Fig. 3) at 4 (A) and 20 weeks (B) postinfection. The size bar in panel A (WT, Lung 4 wks) is 100 μm and applies to all panels in A and B. Filled arrowheads indicate follicles in the spleen, and unfilled arrowheads indicate foci of antigen-presenting cells, most likely macrophages or dendritic cells. More extensive pathology was observed in the lungs of mice infected with wild-type *M. tuberculosis* compared to that of the *mel2* mutant. The spleens of mice infected with wild-type *M. tuberculosis* display foci of antigen-presenting cells at 4 weeks postinfection, whereas mice infected with the *mel2* mutant display less defined foci only at 20 weeks postinfection. (C) Acid-fast stains were used to localize the bacteria in wild-type *M. tuberculosis*- and *mel2* mutant-infected tissues. The size bar in panel C (WT, Lung 4 wks) is 50 μm and applies to all panels in panel C. Although there were fewer granulomas in the lungs of the *mel2* mutant-infected mice, almost double the number of bacteria was present within them. The foci of antigen-presenting cells in the spleen contain similar numbers of bacteria in both wild-type and *mel2* mutant-infected mice, but these foci and the bacteria that they contain were only found in the spleens of *mel2* mutant-infected mice at 20 weeks and were not present at 4 weeks postinfection.

wild type, possibly affecting growth in the lungs. In experiment 1 and two subsequent pilot infection studies (data not shown), we found that, although nearly identical concentrations of bacteria were used for aerosolization, the *mel2* mutant always seeded the lungs at higher levels than the wild type. Although we remain uncertain of the reasons for this phenomenon, we corrected for seeding efficiency by reducing the concentration of the *mel2* mutant used for infection by twofold, resulting in nearly identical numbers (±25%) of bacteria initially seeding the lungs at day 1 in experiments 2 and 3. Using these conditions, we found that when day 1 bacterial numbers are similar, the *mel2* mutant does not differ from the wild type in survival/growth in the lung during the first 4 weeks postinfection (Fig. 3) but does display a defect in persistence ($P < 0.001$). Interestingly, bacterial numbers in the spleen at 4 weeks were less for the *mel2* mutant than the wild type ($P < 0.001$ in experiment 2 and $P < 0.05$ in experiment 3), suggesting that *mel2* plays a role in the dissemination and/or susceptibility to extrapulmonary immune components. These observations suggest that the *M. tuberculosis mel2* locus plays a role in persistence within the lung and dissemination to or survival within extrapulmonary tissues.

The *mel2* mutation affects pathogenesis in mice. Tissues from the lungs and spleens of mice infected with wild-type *M. tuberculosis* and the *mel2* mutant were examined for pathological changes at 4 and 20 weeks postinfection. Hematoxylin and eosin sections were coded for blinded examination and scored for the degree of inflammation and mixed lymphocytic infiltrates, and the numbers of granulomas were quantified for each section. We found that at 4 weeks postinfection, the lungs of *mel2* mutant-infected mice display fewer granulomas (mean, 2 ± 1) than do those of mice infected with the wild type (mean, 5 ± 2). Those granulomas present were smaller and contained fewer lymphocytes, foamy macrophages, and giant cells than granulomas in the wild-type lungs (Fig. 4). Inflammation was less pronounced, with less extensive tissue damage and consolidation resulting in more numerous intact alveoli in regions near granulomas. At 20 weeks postinfection, this pattern is more obvious with more discrete and smaller granulomatous regions present in lungs infected with the *mel2* mutant and nearly normal tissue surrounding the granulomas. Ziehl-Neelsen stains of these same tissues revealed that the lung granulomas of mice infected with the wild type and the *mel2 M. tuberculosis* mutant contain similar numbers or, possibly, the granulomas of mice infected with the *mel2* mutant contain somewhat more bacteria than do those of mice infected with the wild type. These observations correspond well with the

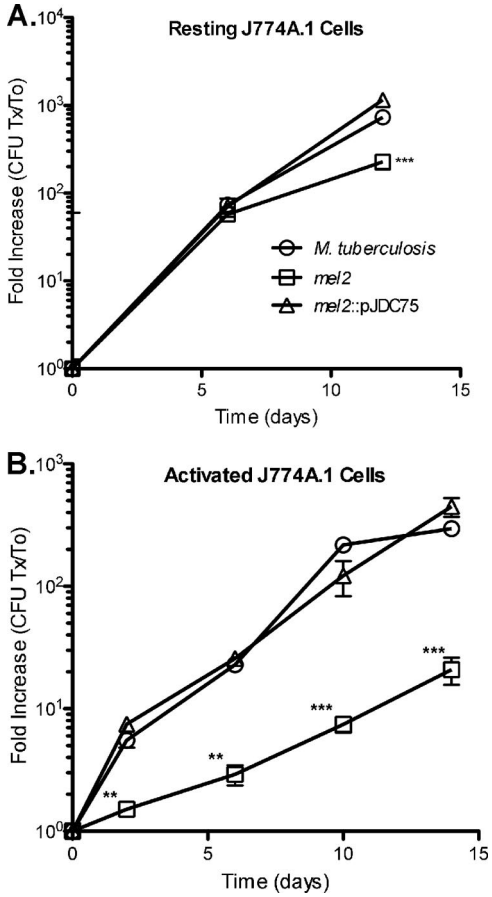

FIG. 5. Growth of wild-type *M. tuberculosis*, *mel2*, and *mel2*-complementing (*mel2*::pJDC75) strains in resting (A) and activated (B) J774A.1 cells. Data and error bars represent the means and standard deviations, respectively, of the results from triplicate samples in a representative experiment carried out three times with similar results. Two asterisks indicate a P of <0.01 and three asterisks indicate a P of <0.001, which are significantly different from that for wild-type *M. tuberculosis*.

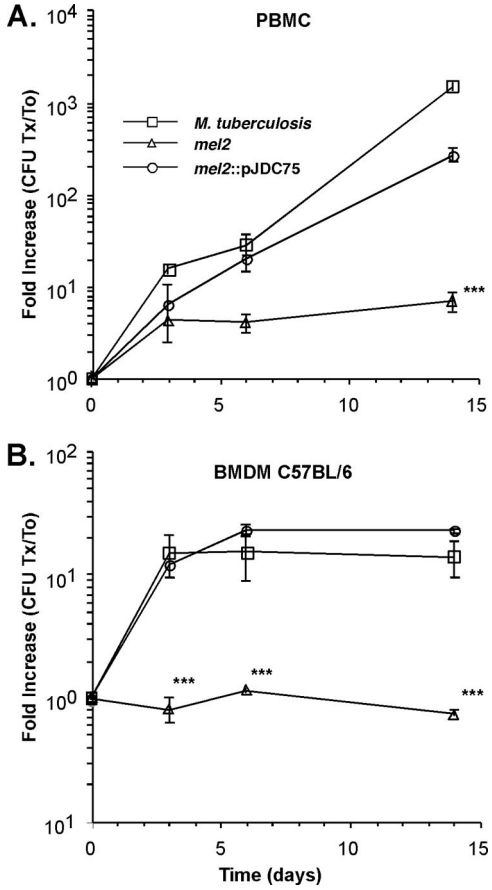

FIG. 6. Growth of wild-type *M. tuberculosis*, *mel2*, and *mel2*-complementing (*mel2*::pJDC75) strains in activated human PBMC (A) and activated strain C57BL/6 murine BMDM (B). Data and error bars represent the means and standard deviations, respectively, of the results from triplicate samples in a representative experiment carried out three times with similar results. Three asterisks indicate a P of <0.001, which is significantly different from that for wild-type *M. tuberculosis*.

presence of similar numbers of bacteria by CFU but with fewer granulomas in the *mel2* mutant-infected mice than in the wild-type-infected mice. The spleens of mice infected with the *mel2* mutant displayed few periarteriolar foci of macrophages or antigen-presenting cells within T follicles compared with those of mice infected with the wild type at 4 weeks postinfection (Fig. 4). However, by 20 weeks postinfection the numbers of such foci in mice infected with the mutant were similar to those of mice infected with the wild type, though in the case of the *mel2* mutant-infected mice, the foci were less circumscribed. Acid-fast bacteria were usually present within what appeared to be antigen-presenting cells in these foci when they were observed, suggesting that *M. tuberculosis* travels to the spleen within these cells. These data suggest that the *mel2* mutant displays decreased lung pathology and delayed dissemination to the spleen.

Survival of *M. tuberculosis* in activated macrophages depends upon *mel2*. The observation that *mel2* is not required initially to combat the innate immune response after infection by aerosol with *M. tuberculosis* but is required for persistence suggests that *mel2* allows *M. tuberculosis* to survive once an acquired immune response is generated or for the metabolic transition to a dormant state necessary for persistence. Since *M. tuberculosis* is thought to grow and replicate primarily within macrophages, we first examined the role of acquired immunity in the elimination of the *mel2* mutant during persistence in the lung by evaluating the susceptibility of this mutant to resting and activated macrophages (Fig. 5). Although the growth/survival of the *mel2* mutant at 12 days in resting murine J774A.1 macrophages is somewhat less than that of the wild type ($P < 0.01$), it is possible that this observation is due to the intrinsic activation of these macrophages by *M. tuberculosis* infection itself, since no difference in growth/survival is observed at 6 days. Growth in activated macrophages is defective for the *mel2* mutant as early as 2 days postinfection up to 14 days ($P < 0.01$). The growth of the *mel2* mutant was also examined in primary cells from humans and mice to determine whether this growth defect was due to a specific characteristic of the J774A.1 cell line, murine cells or generally applicable to all macrophages (Fig. 6). The *mel2* mutant displays a more severe growth defect ($P < 0.001$) in both human PBMC and

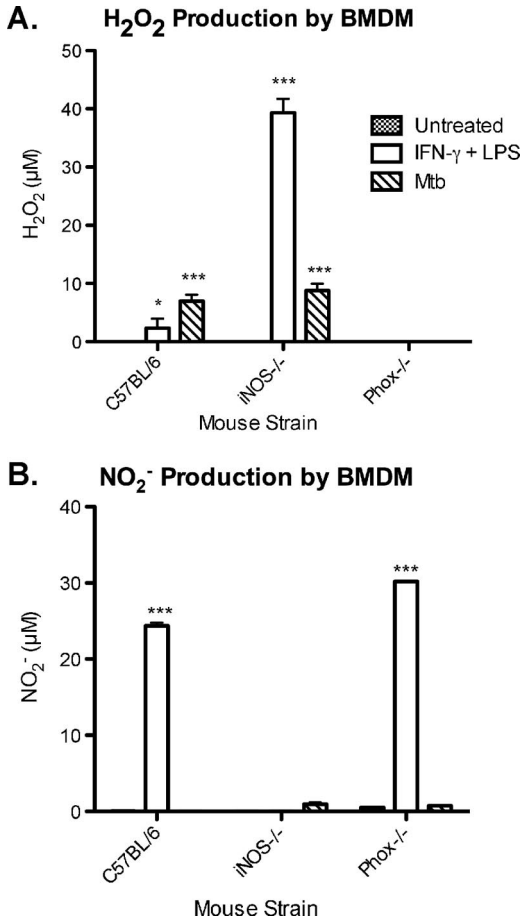

FIG. 7. Production of H_2O_2 (A) and NO_2^- (B) by BMDM from C57BL/6, iNOS$^{-/-}$, and Phox$^{-/-}$ mice untreated and treated with murine IFN-γ plus LPS or *M. tuberculosis*. Data and error bars represent the means and standard deviations, respectively, of the results from quadruplicate samples in a representative experiment carried out two times with similar results. Three asterisks indicate a P of <0.001 and one asterisk indicates a P of <0.05, which are significantly different from that for untreated BMDM from the same mouse strain.

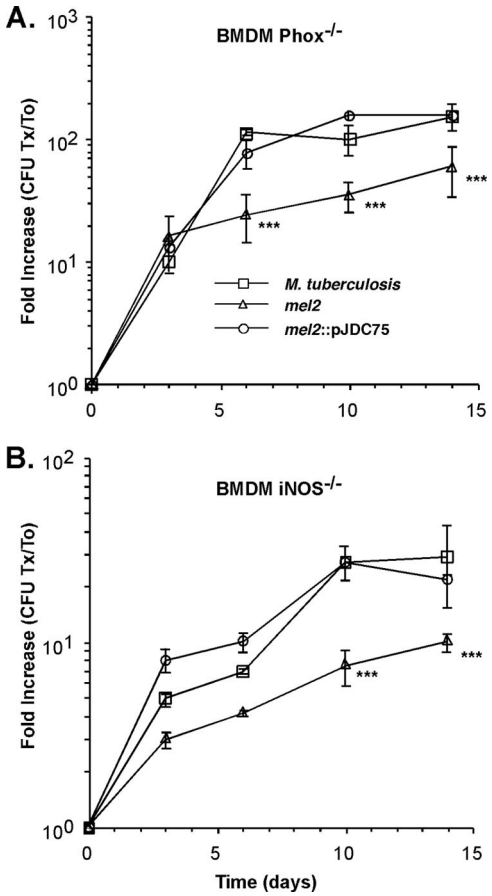

FIG. 8. Growth of wild-type *M. tuberculosis*, *mel2*, and *mel2*-complementing (*mel2*::pJDC75) strains in activated strain C57BL/6 genetic background Phox$^{-/-}$ (A) and iNOS$^{-/-}$ (B) murine BMDM. Data and error bars represent the means and standard deviations, respectively, of the results from triplicate samples in a representative experiment carried out two times with similar results. Three asterisks indicate a P of <0.001, which is significantly different from that for wild-type *M. tuberculosis*.

C57BL/6 mouse BMDM than was observed in the J774A.1 macrophage cell line, suggesting that the *mel2* locus is important for the interaction of *M. tuberculosis* with primary activated macrophages and that the J774A.1 cells are less discriminatory for this phenotype.

The *mel2* locus is important for ROS and RNS resistance. There are several potential mechanisms by which *mel2* could allow the growth of *M. tuberculosis* in activated macrophages, resistance to ROS, resistance to RNS, resistance to both, resistance to other bactericidal pathways, or modification of cellular trafficking pathways. We first chose to determine the role of ROS and RNS in the *mel2* phenotype, since the *mel2* mutant is more susceptible to ROS in culture. We chose primary BMDM for these studies because of the clear phenotype that *mel2* displays in these cells. We measured the levels of production of ROS and RNS by wild-type C57BL/6, iNOS$^{-/-}$, and Phox$^{-/-}$ BMDM with and without activation with IFN-γ plus LPS or in the presence of *M. tuberculosis* (Fig. 7). Both activation with IFN-γ plus LPS and *M. tuberculosis* induce significant levels of ROS in wild-type and iNOS$^{-/-}$ BMDM but not Phox$^{-/-}$ BMDM ($P < 0.001$). However, RNS were only induced after activation with IFN-γ plus LPS in wild type and Phox$^{-/-}$ BMDM but not iNOS$^{-/-}$ BMDM ($P < 0.001$). Interestingly, the levels of ROS produced by iNOS$^{-/-}$ mice after activation were more than four times higher than those of the wild type, suggesting that the absence of iNOS results in the compensatory overproduction of ROS after activation. We found that the *mel2* mutant still displays a defect in both Phox$^{-/-}$ and iNOS$^{-/-}$ activated BMDM ($P < 0.001$), though this defect is less obvious in both cell types than in wild-type BMDM (Fig. 8). These data provide evidence that *mel2* plays a role in resistance to both ROS and RNS in activated macrophages.

***mel2*-mediated ROS resistance is involved in dissemination.** Although our observations indicate that *mel2* is important for resistance to ROS and RNS in activated macrophages in vitro, it is unclear how ROS and RNS production impact the kinetics of infection, since *M. tuberculosis* initially interacts with resting macrophages that may not produce high levels of ROS and

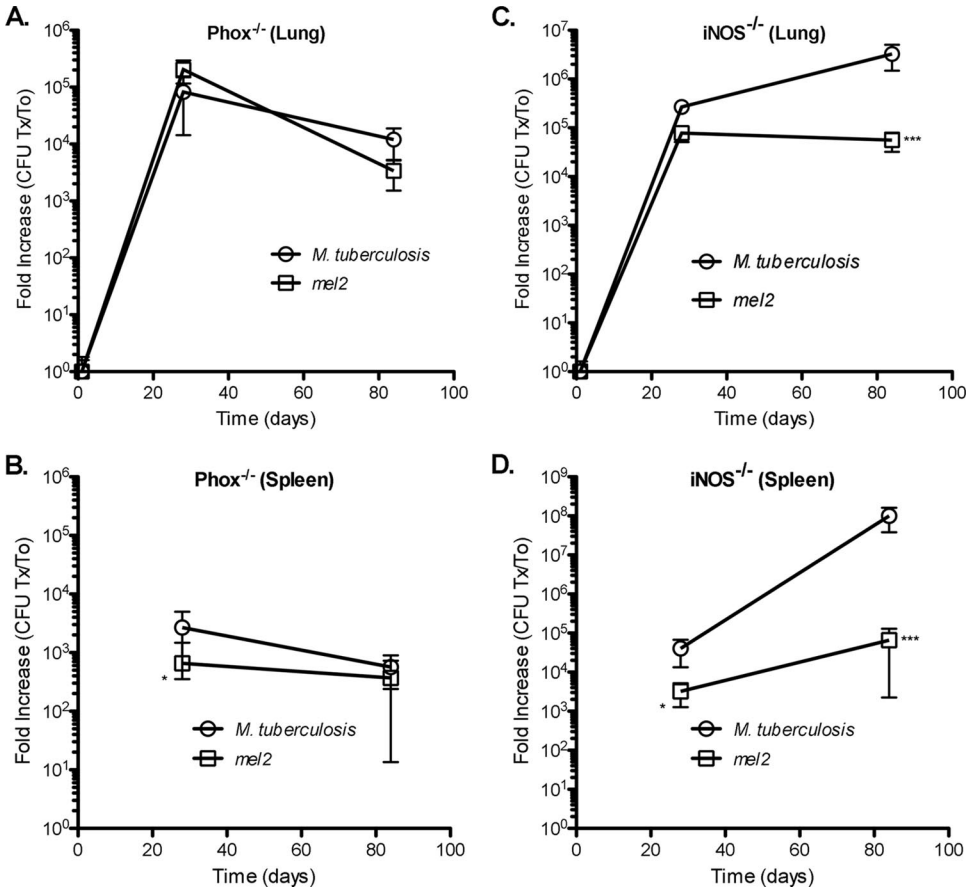

FIG. 9. Growth and persistence of *mel2* mutant in Phox$^{-/-}$ (A, B) and iNOS$^{-/-}$ (C, D) mice. Data shown are the means and standard deviations of the results from four animals in each group for each experiment. The increase is calculated for both the lung and spleen based on the initial numbers of bacteria that seeded the lungs on day 1 after low-dose aerosol infection. An asterisk indicates a P of <0.05 and three asterisks indicate a P of <0.001, which are significantly different from that for wild-type *M. tuberculosis* in the same organ.

RNS and only subsequently is faced with a strong immune response. We evaluated the kinetics of infection in Phox$^{-/-}$ and iNOS$^{-/-}$ mice by wild-type *M. tuberculosis* and the *mel2* mutant using the low-dose aerosol infection model (Fig. 9). We found that in the lungs of Phox$^{-/-}$ mice, the *mel2* mutant behaves similarly to the wild type. Although there is an initial defect in the numbers of bacteria in the spleens of Phox$^{-/-}$ mice, this defect is modest at 4 weeks ($P < 0.05$) and is not significant at later time points. In contrast, iNOS$^{-/-}$ mice display a difference between the *mel2* mutant and the wild type in the lung and spleen very similar to that seen in C57BL/6 mice ($P < 0.001$). These observations suggest that *mel2* is more important for resistance to ROS than RNS and that resistance to ROS and RNS is important for dissemination to the spleen.

***mel2* is involved in the protection of tuberculosis from lipid oxidation.** If the primary role of *mel2* is in the protection of *M. tuberculosis* from ROS, treatment with H_2O_2 should affect not only bacterial viability but also the levels of oxidized molecules within the bacterial cell. We examined the levels of oxidized lipids in the form of LPO within *M. tuberculosis* and the *mel2* mutant after treatment with ROS (Fig. 10). Under normal growth conditions, the *mel2* locus does not affect the basal levels of LPO in *M. tuberculosis*. In contrast, in the presence of ROS, represented by H_2O_2 and t-BOOH, an organic peroxide that decomposes to alkoxyl and peroxyl radicals as well as H_2O_2, the levels of LPO are higher in the *mel2* mutant than in wild-type *M. tuberculosis* ($P < 0.01$). Under the conditions used, despite the fact that the levels of H_2O_2 cause very little loss of viability in wild-type *M. tuberculosis* (Fig. 2), the levels of LPO are significantly higher in all strains than in the untreated bacteria ($P < 0.001$). These observations demonstrate that *M. tuberculosis* is susceptible to the oxidative damage of lipids and that the *mel2* locus is involved in the prevention or repair of ROS-mediated lipid oxidation.

DISCUSSION

These studies found that the *M. tuberculosis mel2* locus, which is a member of a new class of bioluminescence-related genes present in numerous nonbioluminescent pathogens and symbionts, plays a role in *M. tuberculosis* persistence and dissemination in the mouse model of infection. Both of these functions during *M. tuberculosis* pathogenesis may be the result of effects on susceptibility to ROS and RNS produced by host cells that are responding to infection. Overall, the mechanism by which *mel2* affects persistence during pathogenesis is primarily through resistance to ROS and secondarily to RNS. This mechanism is supported by the following four different

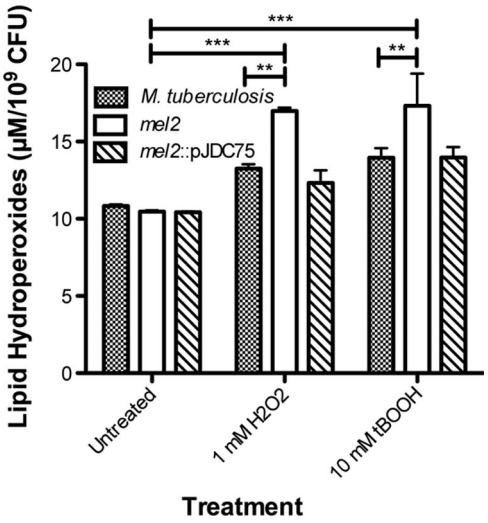

FIG. 10. LPO present in wild-type *M. tuberculosis*, *mel2*, and *mel2*-complementing (*mel2*::pJDC75) strains after treatment with ROS. Bacteria were treated with H_2O_2 or tert-butyl peroxide for 16 h. Data shown are the means and standard deviations of the results from triplicate samples in a representative experiment. Statistics shown are for comparison between the untreated and treated groups as well as the *mel2* mutant compared to wild-type *M. tuberculosis* strains within each group. Two asterisks indicate a P of <0.01 and three asterisks indicate a P of <0.001, which are significantly different.

pieces of evidence: (i) our previous observations in *M. marinum* (61, 62), (ii) the increased susceptibility of the *mel2* mutant to ROS, (iii) the recovery of a wild-type phenotype of the *mel2* mutant in Phox$^{-/-}$ mice, and (iv) the higher levels of ROS-mediated LPO within the bacterial cell. Interestingly, no greater role for *mel2* in resistance to ROS was observed in *M. marinum*, suggesting that there are differences in the mechanisms by which *M. tuberculosis* and *M. marinum* resist ROS and RNS. This is not surprising, since the *M. tuberculosis oxyR* gene, a key regulator of the response to ROS and RNS in other bacterial species, is inactivated but in *M. marinum* is intact (17, 59). This difference impacts the expression of other important genes, including *ahpC* (60), which is involved in resistance to peroxynitrite, a product of nitric oxide and hydrogen peroxide. These observations, along with the data obtained in the current study, suggest that *mel2* is important for resistance to ROS produced as part of an acquired immune response.

An unexpected and intriguing observation that we made in this study is that *mel2* affects the dissemination of *M. tuberculosis* to the spleen. In all cases, including wild-type C57BL/6, Phox$^{-/-}$, and iNOS$^{-/-}$ mice, the dissemination of the *mel2* mutant to the spleen is delayed compared to that of wild-type *M. tuberculosis*. The two most likely possibilities that could explain why *mel2* affects dissemination are that extrapulmonary tissues have a greater ability to produce ROS and RNS at early time points postinfection or that *mel2* is involved in the repair of bacterial components that play a role in dissemination. The first possibility, that extrapulmonary sites have greater bactericidal activity, is consistent with data suggesting that alveolar macrophages are less bactericidal than peritoneal blood monocyte-derived macrophages or PBMC (14, 48). This difference in bactericidal activity in different tissues may be a consequence of the need to prevent hyperresponsiveness in pulmonary tissues, which is thought to be controlled primarily by alveolar macrophages themselves (35, 64). The second possibility, that *mel2* is involved in the repair of *M. tuberculosis* components involved in dissemination, is consistent with our observation that the overexpression or mutation of *mel2* in *M. marinum* affects the infection of macrophages (23). On the surface, it may seem perplexing as to why a locus involved in susceptibility to ROS would affect host-cell infection, but considering that this phenotypic effect may be indirect through the repair of oxidized lipids at the mycobacterial surface that are involved in host cell interactions, this effect might have been anticipated. The repair of oxidized lipids by proteins encoded by *mel2* could occur through a combination of epoxide hydrolase (*melH*) repair of lipid oxidative damage in the form of lipid epoxides and oxidoreductase activities (*melGIK*) reducing the LPO generated through the use of reduced flavin mononucleotide, flavin adenine dinonucleotide, and NADH as cofactors (62). Considering that the *mel2* locus appears to play a primary role in resistance to ROS and the degree of defect in dissemination to the spleen is similar for both Phox$^{-/-}$ and iNOS$^{-/-}$ mice, it is likely that the role of *mel2* in host cell infection is the primary factor that leads to an effect on dissemination. Further studies are needed to better differentiate between these possibilities and evaluate how the mechanisms of host cell infection can affect dissemination.

The *mel2* locus does not appear to play a key role during the early stages of infection when the innate immune response is the primary mediator of bactericidal activity. Only after the acquired immune system is active, at 4 weeks postinfection when the production of TNF-α and IFN-γ normally occurs (5, 32, 38), does the persistence of the *mel2* mutant begin to diverge from wild-type *M. tuberculosis*. At this stage of infection, one would expect that *M. tuberculosis* must cope with activated macrophages that have migrated to the lung along with a strong cell-mediated immune response. The role of activated macrophages in the bactericidal mechanism involved in clearing the *mel2* mutant is supported by our tissue culture studies using isolated primary and immortalized macrophages. Although the *mel2* mutant displays a severe defect for growth in activated macrophages, growth in resting macrophages is only reduced at later time points. These observations suggest that the production of higher levels of ROS and/or RNS in activated macrophages is the primary mechanism responsible, since resting macrophages do not produce significant levels of these bactericidal molecules unless bacteria are present. The fact that the Phox$^{-/-}$ activated macrophages and mice display a less obvious phenotype than C57BL/6 or iNOS$^{-/-}$ mice suggests that the production of ROS by activated macrophages is the primary mechanism by which the *mel2* mutant is cleared during the persistent stage of infection in mice.

Another interesting observation made during these studies is that the pathological changes in the lungs and spleen are different between mice infected with wild-type *M. tuberculosis* and the *mel2* mutant. We had not expected differences at 4 weeks postinfection, since the numbers of bacteria present are similar for the two bacterial strains at this time point. However, the level of tissue damage and the numbers of granulomas present were less for mice infected with the *mel2* mutant than for those infected with wild-type *M. tuberculosis*, suggesting

that despite the same numbers of bacteria present, the disease that the *mel2* mutant causes is less severe. At 20 weeks postinfection, it is not surprising that the lung appears to be recovering from infection, since bacterial numbers are usually at least 10-fold lower for the *mel2* mutant than wild-type *M. tuberculosis* by this time point. A portion of the reduced pathology in the lung may result from fewer *mel2* mutant bacteria in the spleen at the 4-week time point, as evidenced by fewer foci of antigen-presenting cells containing bacteria until 20 weeks postinfection and by differences in CFU. This situation may result in a less aggressive stimulation of the host immune response via the spleen, reducing pathology resulting from the host immune response. This effect does not appear to be detrimental to the ability of the host to mount a protective response in the lung, since the *mel2* mutant is cleared more readily after 4 weeks, but it may reduce tissue damage. Reduced pathology and an inability to persist in the lung are desirable characteristics for an attenuated vaccine strain, and although the *mel2* mutant may not be sufficiently attenuated to be practical on its own, a double mutant with another locus and *mel2* may prove an advantageous strategy for design of a vaccine against *M. tuberculosis*, particularly since it would not be attenuated to the point that it is unable to be protective. Studies are ongoing that compare the immune response in the lungs and spleens of *mel2* mutant- and wild-type-infected mice to gain insight into potential differences in immune modulation involving this locus.

The *mel2* locus is required for the persistence of *M. tuberculosis* in mice after 4 weeks postinfection but does not affect growth at earlier time points. This characteristic of the mutant appears to be related to the mechanism by which *M. tuberculosis* resists ROS, a conclusion that is consistent with the similarity of the genes in *mel2* with bioluminescence systems in other bacteria that have also been implicated in resistance to ROS (2–4, 37, 63). The most likely biochemical mechanism for the effect of *mel2* on susceptibility to ROS is through a catalase-like reaction, as has been observed with bioluminescence systems where H_2O_2 is converted to H_2O and light or an oxidized intermediate (66). Since we have not observed light production by *M. tuberculosis* in the presence of H_2O_2 (S. Subbian and J. D. Cirillo, unpublished observations), we favor the involvement of an oxidized intermediate whose precursor is regenerated by the other genes in the *mel2* locus, similarly to luciferin in bioluminescence systems. The kinetics of the *mel2* phenotype and its catalase-like activity could explain an earlier observation that an *M. tuberculosis katG* mutant displays only a transient phenotype, from 2 to 4 weeks postinfection (47), and the presence of *katG* mutant isoniazid-resistant clinical isolates (43, 45). Overall, these observations clarify the mechanisms of ROS resistance during *M. tuberculosis* pathogenesis and suggest that what may appear to be redundant systems can be involved in different stages of disease, with the *mel2* locus playing a role in resistance to ROS during the chronic or persistence stage of infection.

ACKNOWLEDGMENT

This work was supported by grant AI47866 from the National Institutes of Health.

REFERENCES

1. **Bardarov, S., J. Kriakov, C. Carriere, S. Yu, C. Vaamonde, R. A. McAdam, B. R. Bloom, G. F. Hatfull, and W. R. Jacobs, Jr.** 1997. Conditionally replicating mycobacteriophages: a system for transposon delivery to Mycobacterium tuberculosis. Proc. Natl. Acad. Sci. USA **94:**10961–10966.
2. **Barros, M. P., and E. J. Bechara.** 1998. Bioluminescence as a possible auxiliary oxygen detoxifying mechanism in elaterid larvae. Free Radic. Biol. Med. **24:**767–777.
3. **Barros, M. P., and E. J. Bechara.** 2001. Daily variations of antioxidant enzyme and luciferase activities in the luminescent click-beetle *Pyrearinus termitilluminans*: cooperation against oxygen toxicity. Insect Biochem. Mol. Biol. **31:**393–400.
4. **Barros, M. P., and E. J. Bechara.** 2000. Luciferase and urate may act as antioxidant defenses in larval *Pyrearinus termitilluminans* (*Elateridae: Coleoptera*) during natural development and upon 20-hydroxyecdysone treatment. Photochem. Photobiol. **71:**648–654.
5. **Bean, A. G., D. R. Roach, H. Briscoe, M. P. France, H. Korner, J. D. Sedgwick, and W. J. Britton.** 1999. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol *Mycobacterium tuberculosis* infection, which is not compensated for by lymphotoxin. J. Immunol. **162:**3504–3511.
6. **Bloom, B. R., and C. J. L. Murray.** 1992. Tuberculosis: commentary on a reemergent killer. Science **257:**1055–1064.
7. **Chan, J., K. Tanaka, D. Carroll, J. Flynn, and B. R. Bloom.** 1995. Effects of nitric oxide synthase inhibitors on murine infection with *Mycobacterium tuberculosis*. Infect. Immun. **63:**736–740.
8. **Chan, J., Y. Xing, R. S. Magliozzo, and B. R. Bloom.** 1992. Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages. J. Exp. Med. **175:**1111–1122.
9. **Choi, H. S., P. R. Rai, H. W. Chu, C. Cool, and E. D. Chan.** 2002. Analysis of nitric oxide synthase and nitrotyrosine expression in human pulmonary tuberculosis. Am. J. Respir. Crit. Care Med. **166:**178–186.
10. **Cirillo, J. D., S. L. G. Cirillo, L. Yan, L. E. Bermudez, S. Falkow, and L. S. Tompkins.** 1999. Intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances virulence of *Legionella pneumophila*. Infect. Immun. **67:**4427–4434.
11. **Cirillo, J. D., S. Falkow, L. S. Tompkins, and L. E. Bermudez.** 1997. Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. Infect. Immun. **65:**3759–3767.
12. **Cirillo, S. L. G., L. E. Bermudez, S. H. El-Etr, G. E. Duhamel, and J. D. Cirillo.** 2001. *Legionella pneumophila* entry gene *rtxA* is involved in virulence. Infect. Immun. **69:**508–517.
13. **Cohen, M. K., R. A. Bartow, C. L. Mintzer, and D. N. McMurray.** 1987. Effects of diet and genetics on *Mycobacterium bovis* BCG vaccine efficacy in inbred guinea pigs. Infect. Immun. **55:**314–319.
14. **Collins, F. M., C. J. Niederbuhl, and S. G. Campbell.** 1983. Bactericidal activity of alveolar and peritoneal macrophages exposed in vitro to three strains of *Pasteurella multocida*. Infect. Immun. **39:**779–784.
15. **Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme.** 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J. Exp. Med. **178:**2243–2247.
16. **Cooper, A. M., B. H. Segal, A. A. Frank, S. M. Holland, and I. M. Orme.** 2000. Transient loss of resistance to pulmonary tuberculosis in $p47^{phox-/-}$ mice. Infect. Immun. **68:**1231–1234.
17. **Deretic, V., W. Philipp, S. Dhandayuthapani, M. H. Mudd, R. Curcic, T. Garbe, B. Heym, L. E. Via, and S. T. Cole.** 1995. *Mycobacterium tuberculosis* is a natural mutant with an inactivated oxidative-stress regulatory gene: implications for sensitivity to isoniazid. Mol. Microbiol. **17:**889–900.
18. **Difco.** 1984. Difco manual: dehydrated culture media and reagents for microbiology, 10th ed. Difco Laboratories, Detroit, MI.
19. **Dye, C., A. Bassili, A. L. Bierrenbach, J. F. Broekmans, V. K. Chadha, P. Glaziou, P. G. Gopi, M. Hosseini, S. J. Kim, D. Manissero, I. Onozaki, H. L. Rieder, S. Scheele, F. van Leth, M. van der Werf, and B. G. Williams.** 2008. Measuring tuberculosis burden, trends, and the impact of control programs. Lancet Infect. Dis. **8:**233–243.
20. **Dye, C., S. Scheele, P. Dolin, V. Pathania, M. C. Raviglione, et al.** 1999. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. JAMA **282:**677–686.
21. **Edwards, K. M., M. H. Cynamon, R. K. R. Voladri, C. Hager, M. S. DeStefano, K. T. Tham, D. L. Lakey, M. R. Bochan, and D. S. Kernodle.** 2001. Iron-cofactored superoxide dismutase inhibits host responses to *Mycobacterium tuberculosis*. Am. J. Respir. Crit. Care Med. **164:**2213–2219.
22. **Ehrt, S., D. Schnappinger, S. Bekiranov, J. Drenkow, S. Shi, T. R. Gingeras, T. Gaasterland, G. Schoolnik, and C. Nathan.** 2001. Reprogramming of the macrophage transcriptome in response to interferon-gamma and *Mycobacterium tuberculosis*: signaling roles of nitric oxide synthase-2 and phagocyte oxidase. J. Exp. Med. **194:**1123–1140.
23. **El-Etr, S. H., S. Subbian, S. L. Cirillo, and J. D. Cirillo.** 2004. Identification of two *Mycobacterium marinum* loci that affect interactions with macrophages. Infect. Immun. **72:**6902–6913.

24. **Fang, F. C.** 2004. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat. Rev. Microbiol. **2:**820–832.
25. **Flesch, I. E., and S. H. Kaufmann.** 1990. Activation of tuberculostatic macrophage functions by gamma interferon, interleukin-4, and tumor necrosis factor. Infect. Immun. **58:**2675–2677.
26. **Flynn, J. L.** 2006. Lessons from experimental Mycobacterium tuberculosis infections. Microbes Infect. **8:**1179–1188.
27. **Flynn, J. L., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom.** 1993. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. J. Exp. Med. **178:**2249–2254.
28. **Flynn, J. L., M. M. Goldstein, J. Chan, K. J. Triebold, K. Pfeffer, C. J. Lowenstein, R. Schreiber, T. W. Mak, and B. R. Bloom.** 1995. Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. Immunity **2:**561–572.
29. **Flynn, J. L., C. A. Scanga, K. E. Tanaka, and J. Chan.** 1998. Effects of aminoguanidine on latent murine tuberculosis. J. Immunol. **160:**1796–1803.
30. **Glickman, M. S., J. S. Cox, and W. R. Jacobs, Jr.** 2000. A novel mycolic acid cyclopropane synthetase is required for cording, persistence, and virulence of *Mycobacterium tuberculosis*. Mol. Cell **5:**717–727.
31. **Glickman, M. S., and W. R. Jacobs, Jr.** 2001. Microbial pathogenesis of *Mycobacterium tuberculosis*: dawn of a discipline. Cell **104:**477–485.
32. **Hernandez-Pando, R., H. Orozco, K. Arriaga, A. Sampieri, J. Larriva-Sahd, and V. Madrid-Marina.** 1997. Analysis of the local kinetics and localization of interleukin-1 alpha, tumour necrosis factor-alpha and transforming growth factor-beta, during the course of experimental pulmonary tuberculosis. Immunology **90:**607–617.
33. **Heym, B., E. Stavropoulos, N. Honore, P. Domenech, B. Saint-Joanis, T. M. Wilson, D. M. Collins, M. J. Colston, and S. T. Cole.** 1997. Effects of overexpression of the alkyl hydroperoxide reductase AhpC on the virulence and isoniazid resistance of *Mycobacterium tuberculosis*. Infect. Immun. **65:**1395–1401.
34. **Hingley-Wilson, S. M., V. K. Sambandamurthy, and W. R. Jacobs, Jr.** 2003. Survival perspectives from the world's most successful pathogen, *Mycobacterium tuberculosis*. Nat. Immunol. **4:**949–955.
35. **Holt, P. G., J. Oliver, N. Bilyk, C. McMenamin, P. G. McMenamin, G. Kraal, and T. Thepen.** 1993. Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. J. Exp. Med. **177:**397–407.
36. **Jeevan, A., C. T. McFarland, T. Yoshimura, T. Skwor, H. Cho, T. Lasco, and D. N. McMurray.** 2006. Production and characterization of guinea pig recombinant gamma interferon and its effect on macrophage activation. Infect. Immun. **74:**213–224.
37. **Katsev, A. M., G. Wegrzyn, and H. Szpilewska.** 2004. Effects of hydrogen peroxide on light emission by various strains of marine luminescent bacteria. J. Basic Microbiol. **44:**178–184.
38. **Lazarevic, V., D. Nolt, and J. L. Flynn.** 2005. Long-term control of *Mycobacterium tuberculosis* infection is mediated by dynamic immune responses. J. Immunol. **175:**1107–1117.
39. **Li, Z., C. Kelley, F. Collins, D. Rouse, and S. Morris.** 1998. Expression of *katG* in *Mycobacterium tuberculosis* is associated with its growth and persistence in mice and guinea pigs. J. Infect. Dis. **177:**1030–1035.
40. **Liew, F. Y., C. Parkinson, S. Millott, A. Severn, and M. Carrier.** 1990. Tumour necrosis factor (TNF alpha) in leishmaniasis. I. TNF alpha mediates host protection against cutaneous leishmaniasis. Immunology **69:**570–573.
41. **Lyzen, R., and G. Wegrzyn.** 2005. Sensitivity of dark mutants of various strains of luminescent bacteria to reactive oxygen species. Arch. Microbiol. **183:**203–208.
42. **MacMicking, J. D., R. J. North, R. LaCourse, J. S. Mudgett, S. K. Shah, and C. F. Nathan.** 1997. Identification of nitric oxide synthase as a protective locus against tuberculosis. Proc. Natl. Acad. Sci. USA **94:**5243–5248.
43. **Marttila, H. J., H. Soini, P. Huovinen, and M. K. Viljanen.** 1996. *katG* mutations in isoniazid-resistant *Mycobacterium tuberculosis* isolates recovered from Finnish patients. Antimicrob. Agents Chemother. **40:**2187–2189.
44. **Mohan, V. P., C. A. Scanga, K. Yu, H. M. Scott, K. E. Tanaka, E. Tsang, M. M. Tsai, J. L. Flynn, and J. Chan.** 2001. Effects of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis: possible role for limiting pathology. Infect. Immun. **69:**1847–1855.
45. **Musser, J. M., V. Kapur, D. L. Williams, B. N. Krieswirth, D. van Soolingen, and J. D. van Embden.** 1996. Characterization of the catalase-peroxidase gene (*katG*) and *inhA* locus in isoniazid-resistant and -susceptible strains of *Mycobacterium tuberculosis* by automated DNA sequencing: restricted array of mutations associated with drug resistance. J. Infect. Dis. **173:**196–202.
46. **Nathan, C., and M. U. Shiloh.** 2000. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc. Natl. Acad. Sci. USA **97:**8841–8848.
47. **Ng, V. H., J. S. Cox, A. O. Sousa, J. D. MacMicking, and J. D. McKinney.** 2004. Role of KatG catalase-peroxidase in mycobacterial pathogenesis: countering the phagocyte oxidative burst. Mol. Microbiol. **52:**1291–1302.
48. **Nibbering, P. H., M. T. van den Barselaar, J. S. van de Gevel, and R. van Furth.** 1989. Defective intracellular killing of micro-organisms by murine alveolar macrophages. Agents Actions **26:**206–207.
49. **Orme, I. M.** 1988. A mouse model of the recrudescence of latent tuberculosis in the elderly. Am. Rev. Respir. Dis. **137:**716–718.
50. **Orme, I. M.** 2003. The mouse as a useful model of tuberculosis. Tuberculosis (Edinburgh) **83:**112–115.
51. **Orme, I. M.** 2005. Tuberculosis vaccines: current progress. Drugs **65:**2437–2444.
52. **Piddington, D. L., F. C. Fang, T. Laessig, A. M. Cooper, I. M. Orme, and N. A. Buchmeier.** 2001. Cu,Zn superoxide dismutase of *Mycobacterium tuberculosis* contributes to survival in activated macrophages that are generating an oxidative burst. Infect. Immun. **69:**4980–4987.
53. **Pym, A. S., P. Domenech, N. Honore, J. Song, V. Deretic, and S. T. Cole.** 2001. Regulation of catalase-peroxidase (KatG) expression, isoniazid sensitivity and virulence by *furA* of *Mycobacterium tuberculosis*. Mol. Microbiol. **40:**879–889.
54. **Qadoumi, M., I. Becker, N. Donhauser, M. Rollinghoff, and C. Bogdan.** 2002. Expression of inducible nitric oxide synthase in skin lesions of patients with American cutaneous leishmaniasis. Infect. Immun. **70:**4638–4642.
55. **Rees, J.-F., B. De Wergifosse, O. Noiset, M. Dubuisson, B. Janssens, and E. M. Thompson.** 1998. The origins of marine bioluminescence: turning oxygen defence mechanisms into deep-sea communication tools. J. Exp. Biol. **201:**1211–1221.
56. **Rhoades, E. R., and I. M. Orme.** 1998. Similar responses by macrophages from young and old mice infected with *Mycobacterium tuberculosis*. Mech. Ageing Dev. **106:**145–153.
57. **Scanga, C. A., V. P. Mohan, H. Joseph, K. Yu, J. Chan, and J. L. Flynn.** 1999. Reactivation of latent tuberculosis: variations on the Cornell murine model. Infect. Immun. **67:**4531–4538.
58. **Schneemann, M., G. Schoedon, S. Hofer, N. Blau, L. Guerrero, and A. Schaffner.** 1993. Nitric oxide synthase is not a constituent of the antimicrobial armature of human mononuclear phagocytes. J. Infect. Dis. **167:**1358–1363.
59. **Sherman, D. R., P. J. Sabo, M. J. Hickey, T. M. Arain, G. G. Mahairas, Y. Yuan, C. E. Barry III, and C. K. Stover.** 1995. Disparate responses to oxidative stress in saprophytic and pathogenic mycobacteria. Proc. Natl. Acad. Sci. USA **92:**6625–6629.
60. **Springer, B., S. Master, P. Sander, T. Zahrt, M. McFalone, J. Song, K. G. Papavinasasundaram, M. J. Colston, E. Boettger, and V. Deretic.** 2001. Silencing of oxidative stress response in *Mycobacterium tuberculosis*: expression patterns of *ahpC* in virulent and avirulent strains and effect of *ahpC* inactivation. Infect. Immun. **69:**5967–5973.
61. **Subbian, S., P. K. Mehta, S. L. Cirillo, L. E. Bermudez, and J. D. Cirillo.** 2007. A *Mycobacterium marinum mel2* mutant is defective for growth in macrophages that produce reactive oxygen and reactive nitrogen species. Infect. Immun. **75:**127–134.
62. **Subbian, S., P. K. Mehta, S. L. Cirillo, and J. D. Cirillo.** 2007. The *Mycobacterium marinum mel2* locus displays similarity to bacterial bioluminescence systems and plays a role in defense against reactive oxygen and nitrogen species. BMC Microbiol. **7:**4.
63. **Szpilewska, H., A. Czyz, and G. Wegrzyn.** 2003. Experimental evidence for the physiological role of bacterial luciferase in the protection of cells against oxidative stress. Curr. Microbiol. **47:**379–382.
64. **Thepen, T., N. Van Rooijen, and G. Kraal.** 1989. Alveolar macrophage elimination in vivo is associated with an increase in pulmonary immune response in mice. J. Exp. Med. **170:**499–509.
65. **Walker, L., and D. B. Lowrie.** 1981. Killing of *Mycobacterium microti* by immunologically activated macrophages. Nature **293:**69–71.
66. **Watanabe, H., T. Nagoshi, and H. Inaba.** 1993. Luminescence of a bacterial luciferase intermediate by reaction with H_2O_2: the evolutionary origin of luciferase and source of endogenous light emission. Biochim. Biophys. Acta **1141:**297–302.
67. **Weinberg, J. B.** 1998. Nitric oxide production and nitric oxide synthase type 2 expression by human mononuclear phagocytes: a review. Mol. Med. **4:**557–591.
68. **Zhang, X., and D. N. McMurray.** 1998. Suppression of lymphoproliferation by alveolar macrophages in the guinea pig. Tuber. Lung Dis. **79:**119–126.

Editor: A. Camilli